



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Pergolizzi et al.

Serial No.: 08/479,995

Filed: June 7, 1995

For: ANALYTE DETECTION UTILIZING
POLYNUCLEOTIDE SEQUENCES,
COMPOSITION, PROCESS AND KIT
(As Previously Amended)

Group Art Unit: 1631

Ex'r: Ardin H. Marschel, Ph.D.

South Portland, Maine

Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF DR. ALEX A. WALDROP, III

I, Alex A. Waldrop, III, hereby declare as follows:

1. Since 2000, I have been the sole proprietor of my own start-up company having a principal place of business at the Center for Environmental Enterprise (CEE), South Portland, Maine.¹ My present research work focuses on acridine compounds, such as 9-acridinecarbonylimidazole (AcriGlow™ 301), for use in chemiluminescent assays for medical and environmental diagnostics. My professional experience includes research at several organizations, including Maine Medical Center Research Institute, South Portland, Maine (1994 to 2000), IDEXX

¹ CEE is a private, non-profit organization funded by the State of Maine as a business incubator. Located on the campus of Southern Maine Community College, CEE helps new and young firms like my own to commercialize technologies in the environmental field.

Laboratories, Inc., Westbrook, Maine (1992-1993), and Gen-Probe, Inc., San Diego, California (1985-1992) as described in my *curriculum vitae* (cv).² Over the past several years I served as a consultant for companies such as Brims Ness, Capricorn Products, Inc., Maine Standards, and Enzo Biochem, Inc.

2. My education and research experience are listed in my cv. I received my bachelor of science degree (B.S.) from the University of Virginia in 1970, graduating with high distinction (*magna cum laude*). In 1977 I received my doctoral degree (Ph.D.) in biophysics from The Johns Hopkins University, Baltimore, Maryland. While at Johns Hopkins, I trained in the Department of Biophysics as a pre-doctoral fellow in the laboratory of Dr. Michael Beer from 1970-1977. I developed multiple heavy atom stains for electron microscopy of nucleic acids. My doctoral dissertation was titled "Chemical Studies of *bis*(Pyridine)osmate(VI) Esters and the Mercury Enhancement of Osmium Labelling of Polynucleotides" [Dissertation Abstracts International 38 (11-B):5354 + (194 pp.) (1978)]. As a postdoctoral fellow, I worked in the laboratory of Dr. David C. Ward at Yale University, New Haven, Connecticut from 1977-1980. While at Yale I used reactions with heavy metal intermediates to synthesize detectable non-radioactively modified nucleotides. I contributed to the discovery that these modified nucleotides could be incorporated *in vitro* into nucleic acids for use as non-radioactive nucleic acid probes. This discovery led directly to the development of several non-radioactively modified nucleotides and nucleotide analogs which are used for *in situ* gene and nucleic acid detection. These modified nucleotides and nucleotide analogs and their use in detection processes are described in several U.S. patents (Nos. 4,711,955; 5,328,824; 5,449,767; and 5,476,928). I am one

² Copy attached as Exhibit 1.

of three inventors listed on these patents.³ These modified nucleotides and nucleotide analogs include biotinylated nucleotides and other labeled nucleic acid compositions which have been sold commercially for years.

3. After my postdoctoral work, I was Assistant Professor of Chemistry at the University of Virginia, Charlottesville, Virginia, from 1980-1982. While working in the UVA Department of Chemistry, I taught undergraduate biophysical chemistry. I also prepared nucleotide derivatives of tubercidin and characterized allylamine derivatives. From 1982-1985, I was Research Associate in the Department of Microbiology at UVA where I worked on several projects including the development of a new DNA sequencing method and a gel filtration method for nucleotide purification and desalting, and the synthesis of a series of 5'-thymidine triphosphate derivatives and a dUTP analog containing an ethylenediamine-tetraacetic acid (EDTA) group.

4. I am the author of five scientific publications and I am also an inventor on seven U.S. patents, including the four patents referenced in paragraph 2 above.

5. Enzo Life Sciences, Inc. has asked me as its scientific consultant to review recent portions of the prosecution history of United States Patent Application Serial No. 08/479,995, filed on June 7, 1995 ("the '995 Application") in the name of Robert G. Pergolizzi, *et al.* The title of the '995 Application is "Analyte Detection Utilizing Polynucleotide Sequences,

³ All four of these U.S. patents name David C. Ward, Pennina R. Langer and Alexander A. Waldrop, III, as co-inventors. U.S. Patent No. 4,711,955 is titled "Modified Nucleotides and Methods of Preparing and Using Same" and it issued on December 8, 1987. U.S. Patent No. 5,328,824 is titled "Methods of Using Labeled Nucleotides" and it issued on July 12, 1994. U.S. Patent No. 5,449,767 is titled "Modified Polynucleotides and Methods of Preparing Same," having issued on September 12, 1995. The fourth, U.S. Patent No. 5,476,928, is titled "Modified Nucleotides and Polynucleotides and Complexes Formed Therefrom," and it issued on December 19, 1995.

Composition, Process and Kit." Included for my review were the following documents and materials:

- the original specification [U.S. Patent Application Serial No. 06/491,929, filed on May 5, 1983];
- an Office Action dated February 17, 2004;
- two documents cited in the February 17, 2004 Office Action:
 - T. Maniatis, E. F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982, pages 51-54; and
 - Pennina R. Langer, Alex A. Waldrop and David C. Ward, "Enzymatic synthesis of biotin-labeled nucleotides: Novel nucleic acid affinity probes," Proc. Natl. Acad. Sci. (USA), Volume 78, pages 6633-6637 (November 1981) (for the sake of simplicity, hereinafter referred to as "my 1981 paper").^{4,5}

I have also reviewed two Responses that were filed in the '995 Application, including:

- Applicants' May 28, 2004 Amendment Under 37 C.F.R. §1.115; and
- Applicants' August 20, 2004 Supplemental Amendment To Their May 28, 2004 Amendment Under 37 C.F.R. §1.115.

In connection with the August 20, 2004 Supplemental Declaration, I also reviewed a set of the pending claims in the '995 Application, including claims 506 and 510. I am being compensated for my review and for making this Declaration.

⁴ Copy attached as Exhibit 2.

⁵ I am the same Alex A. Waldrop who is listed as a co-author on this publication. At the time of publication in 1981, I was Assistant Professor of Chemistry at the University of Virginia, Charlottesville, Virginia. Before that, I was at Yale University working in collaboration with Dr. David C. Ward and Pennina R. Langer, also both of Yale.

6. I understand that in the February 17, 2004 Office Action, the Patent Examiner rejected claims 506 and 510, and one other claim, 509, as being anticipated by my 1981 paper.^{6,7} Claims 506 and 510 in the '995 Application reads as follows:

506. A polynucleotide sequence covalently attached to an antibody.

510. A polynucleotide sequence covalently attached to a hormone.

7. As set forth above and in my cv, I am a chemist with substantial experience and background in nucleic acid chemistry and protein chemistry. My knowledge, background, training and experience in nucleic acid chemistry encompasses nucleic acid modifications, including labeling nucleic acids for use in hybridization and detection assays. In protein chemistry, I am familiar with protein modifications and labeling proteins, enzymes and antibodies for use in protein detection. I am familiar with several nucleic acid and protein detection formats and with nucleic acid and protein probe technology in general. My professional and academic career involves

⁶ The Patent Examiner wrote on page 6 in the February 17, 2004 Office Action:

Langer et al. discloses polynucleotides of DNA or RNA with biotin covalently attached which covalent attachment also results in antibody attachment which anticipates the above listed instant claims. Such an antibody is also a receptor for biotin binding as required in certain instant claim 510. See the entire document and especially page 6635, first column, second full paragraph.

⁷ The portion on page 6635 in my 1981 paper cited by the Patent Examiner in the Office Action reads as follows:

The second line of evidence for biotin substitution is that only polynucleotides synthesized in the presence of biotin-labeled nucleotides are immunoprecipitated when treated with purified anti-biotin antibodies and then with formalin-fixed *Staphylococcus* (Table 2). Although the amount of biotin-labeled polymer found in the immune precipitate is dependent on the antibody concentration and time of incubation, under optimum conditions, >90% of the product can be immunoprecipitated, even when present in subnanogram quantities. Significantly, the results in Tables 1 and 2 show that the biotin molecule can be recognized by avidin and anti-biotin antibodies when the DNA is still in a double-stranded form. Parallel experiments (not shown) indicate that biotin-labeled DNA•RNA hybrids and RNA duplexes behave similarly. These observations suggest that immunological and affinity methods could be used for probe detection (or isolation) following standard hybridization procedures.

extensive research exploring the modifications and labeling of nucleic acids for use as probes in hybridization and detection assays. To a lesser extent, I have performed research with proteins, polypeptides, hormones, enzymes and antibodies, again, for detection purposes. One of my more recent areas of research is chemiluminescence and assays using chemiluminescent reagents to detect a wide variety of substances including nucleic acids, proteins and other biomolecules.

8. Based upon my training, background and experience, I believe that at the time the '995 Application was filed in May 1983, the relevant art to the subject matter being claimed would have included many if not most of the following areas: modifications of nucleic acids and proteins, nucleic acid and protein synthesis and labeling, nucleic acid hybridization, protein-protein interactions, substrate recognition by protein, and detection. I consider myself to possess the level of skill, knowledge, training and experience of at least a person of ordinary skill in the art to which the subject matter of present claims 506 and 510 pertain.⁸

9. I have been informed that in order to anticipate a patent claim, the law requires that the four corners of a single, prior art document must describe every material element of the claimed invention, either expressly or inherently, such that a person of ordinary skill in the art could practice the invention without undue experimentation. I have also been informed that an anticipating document must describe the subject matter being sought with sufficient clarity and detail to establish that the subject matter existed and that its existence was recognized by persons of ordinary skill in the field of the invention.

⁸ I understand that claim 509 was canceled in Applicants' August 20, 2004 Supplemental Amendment. For purposes of making this Declaration, my statements are confined to claims 506 and 510.

10. As a person of ordinary skill in the art, it is my opinion that my 1981 paper does not anticipate claims 506 and 510 because at least one material claim element is clearly lacking in the paper. Unlike claims 506 and 510, my 1981 paper does not disclose the *covalent attachment* of a polynucleotide sequence to an antibody or to a hormone; it only describes the attachment of an antibody to a polynucleotide by means of *noncovalent* binding of one antibody (antibiotin) to its corresponding antigen (biotin). I believe, therefore, that the subject matter of claims 506 and 510 is novel over my 1981 paper. My reasons for making this statement are set forth in the paragraphs that follow below.

11. It is textbook chemistry that the interactions between chemical molecules fall into one of two camps, covalent interactions and noncovalent interactions. It is also textbook chemistry that covalent bonds and noncovalent bonds are quite different from each other. A covalent bond is typically defined as "a bond formed between two atoms and consisting of one or more shared pairs of electrons such that one electron in a pair is donated by each of the two bonded atoms."⁹ A noncovalent bond is every bond that is not covalent. A good description of covalent and noncovalent bonds is provided by Garrett and Grisham:

Covalent bonds hold atoms together so that molecules are formed. In contrast, **weak chemical forces** or **noncovalent bonds** (hydrogen bonds, van der Waals forces, ionic interactions, and hydrophobic interactions) are intramolecular or intermolecular attractions between atoms. None of these forces, which typically range from 4 to 30 kJ/mol, are strong enough to bind free atoms together (Table 1.3). The average kinetic energy of molecules at 25°C is 2.5 kJ/mol, so the energy of weak forces is only several times greater than the dissociating tendency due to thermal motion of molecules. Thus, these weak forces create interactions that are constantly forming and breaking at physiological temperature, unless by cumulative number they impart stability to the structures generated by their collective

⁹ Stenesh, J., Dictionary of Biochemistry and Molecular Biology, Second Edition, John Wiley & Sons, New York, 1989, page 105; copy attached as Exhibit 3.

action. These weak forces merit further discussion because their attributes profoundly influence the nature of the biological structures they build.¹⁰ [emphasis in original]

As described above, a combination of weak interactions can cumulatively yield strong interactions, and a good example is the binding that occurs between biotin and avidin, or between biotin and an antibody, such as Immunoglobulin G (IgG).

12. There are many interactions and processes in biochemistry that involve the weak chemical forces or noncovalent bonds. Garrett and Grisham explain:

Weak chemical forces underlie the interactions that are the basis of biomolecular recognition. It is important to realize that because these interactions are sufficiently weak, they are readily reversible. Consequently, biomolecular interactions tend to be transient; rigid, static lattices of biomolecules that might paralyze cellular activities are not formed. Instead, a dynamic interplay occurs between metabolites and macromolecules, hormones and receptors, and all the other participants instrumental to life processes. This interplay is initiated upon specific recognition between complementary molecules and ultimately culminates in unique physiological activities. Biological function is achieved through mechanisms based on structural complementarity.¹¹

Among the examples of biological processes that rely on noncovalent bonds are the reaction of an antigen with its antibody, the binding of many hormones and drugs to nucleic acids and proteins, the reading of the genetic message (codon-anticodon recognition), and the secondary structure of proteins and nucleic acids (including denaturation and renaturation).

¹⁰ From Reginald H. Garrett and Charles M. Grisham's Biochemistry, Third Edition, Brooks/Cole, a division of Thomson Learning, Inc., Belmont, CA, 2005[sic], Chapter 1, page 13; copy of pages 13-19 attached as Exhibit 4.

¹¹ Exhibit 4, paragraph bridging pages 16 and 17.

13. In my 1981 paper, we synthesized nucleotide analogs in which biotin was directly and covalently bonded via a linker arm to the 5-position of the uridine ring. In contrast to the prior art, we disclose on the first page¹² that:

[b]iotin directly attached to a nucleotide that functions as an efficient polymerase substrate would be more versatile, both in the experimental protocols and in the detection methods that could be used.

In the very next paragraph that carries over to the right column, we continue:

We have synthesized a number of nucleotide analogs that contain potential probe determinants (e.g., biotin, iminobiotin, and 2,4-dinitrophenyl groups) covalently attached to the pyrimidine or purine ring in the hope that one of them might prove to be a useful affinity reagent. This report describes the synthesis of biotin-labeled derivatives of UTP and dUTP (1 and 2, respectively) that are substrates for RNA or DNA polymerases. The properties of the resulting biotin-substituted polynucleotides appear to satisfy the basic criteria required of a good affinity probe. [emphasis added]

In my 1981 paper (page 6635, left column, second full paragraph¹³), we disclose that "the biotin molecule can be recognized by avidin and *antibiotin antibodies* . . ." This recognition results in the noncovalent binding of the antibody to the biotin. In the case of my 1981 paper, the antibiotin, IgG, was used as the antibody. See Table 2, page 6636, left column. See also MATERIALS AND METHODS, page 6633, right column ("The following enzymes and reagents were gifts: . . . rabbit antibiotin serum (F. Harmon).")

14. As a person of ordinary skill in the art, it is my opinion that one could not practice the invention described in claims 506 and 510 from a reading of my 1981

¹² Exhibit 2, page 6633, left column, last sentence in the first paragraph, continuing through the next paragraph bridging the left and right columns.

¹³ This is the same paragraph cited by the Patent Examiner on page 6 in the July 14, 2004 Office Action, and also quoted in Footnote 7 above.

paper because it is clear that the binding interactions between biotin and the anti-biotin IgG were *noncovalent*. Because the binding interactions between biotin and IgG were noncovalent, my 1981 paper clearly fails to disclose *covalent antibody attachment* to a polynucleotide sequence, as called for by claim 506.¹⁴ Moreover, it is my opinion as a person of ordinary skill in the art that my 1981 paper does not describe the covalent attachment of a polynucleotide sequence to an antibody to establish that the subject matter of claim 506 existed and was recognized in the field. With respect to claim 510, I would like to point out that my 1981 paper fails to disclose any hormones, let alone any covalent attachment of a hormone to a polynucleotide sequence.

15. With regard to the statements in the anticipation rejection,¹⁵ I agree that my 1981 paper discloses polynucleotides of DNA or RNA with biotin covalently attached. I respectfully and strongly disagree, however, that the covalent attachment of biotin to the polynucleotide in my 1981 paper resulted in covalent antibody attachment as required by claim 506, or that it resulted in covalent hormone attachment as required by claim 510. To assert that biotin is covalently attached to a polynucleotide, and that such covalent attachment results in covalent antibody attachment which meets the material elements of claims 506 and 510 is an erroneous and unreasonable characterization of my 1981 paper. As explained in

¹⁴ On top of that, because of its sheer size, covalently attaching the IgG antibody disclosed in my 1981 PNAS paper to a nucleotide for incorporation into a polynucleotide by a polymerase would have been deemed impractical if not unworkable. See Abstract and first two paragraphs on page 6633 in my 1981 paper.

¹⁵ February 17, 2004 Office Action, page 6, second full paragraph.

Paragraph 11 above, covalent bonds and noncovalent bonds are very different from each other. Claim 506 recites that a polynucleotide sequence is covalently attached to an antibody, and claim 510 recites that a polynucleotide sequence is covalently attached to a hormone. As explained above, my 1981 paper does not disclose that IgG is covalently attached to a polynucleotide sequence. Instead, my 1981 paper describes the noncovalent attachment of IgG to biotin, which is materially different from the recitation in claims 506 and 510.

16. In summary, and for the reasons given above, I conclude as a person of ordinary skill in the art that the subject matter of claims 506 and 510 is novel over my 1981 paper.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Aug 31, 2004
Date

Dr. Alex A. Waldrop, III
Dr. Alex A. Waldrop, III

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Curriculum Vitae of Alex A. Waldrop, III

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EDUCATION

Ph.D. (Biophysics) 1977, Johns Hopkins University, Baltimore, Maryland,
Thesis Advisor: Dr. Michael Beer,
Thesis Title: "Chemical Studies of
bis(Pyridine)osmate(VI) Esters and the Mercury
Enhancement of Osmium Labelling of Polynucleotides"
Dissertation Abstracts International **38** (11-B):5354+
(194 pp.) (1978);
B.S. (Chemistry) 1970, Magna cum Laude, University of Virginia.

HONORS

Echols Scholar, Phi Eta Sigma, Hugh Miller Spencer Scholarship in
Chemistry, 1970.

PROFESSIONAL MEMBERSHIPS

Alpha Chi Sigma, Sigma Xi, AAAS, AACC, American Chemical Society.

EXPERIENCE

Founder and Principal Scientist, Started Company at Center for
Environmental Enterprise (CEE), 2000 to present. Further characterized
9-Acridinecarbonylimidazole (AcriGlow 301) and its reaction with
peroxide in various buffers and solvents. Examined ways of removing
peroxide impurities from solvents, detergent and polymer solutions.
Tested screening assay for detecting pollutants in environmental water
samples. Served as consultant for Brims Ness, Capricorn Products, Inc.,
Maine Standards, and Enzo Biochem, Inc.

Visiting Scientist, Maine Medical Center Research Institute, 1994 to
2000. Synthesized and characterized modified acridancarboxylic acid
ester. Demonstrated substrate activity with HRPO. Invented and
characterized activated 9-acridinecarboxylic acid derivatives.
Demonstrated high sensitivity assay of glucose oxidase and alkaline
phosphatase. HPLC of acridine derivatives. HPLC of synthetic
oligonucleotides.

Research Scientist, IDEXX Laboratories, Inc., 1992 - 1993.
Optimization of HRPO assay systems.

Staff Scientist, Gen-Probe, Inc., 1985 - 1992. Synthesized and designed
acridinium esters. Helped design linker arms, optimize detection of

acridinium esters, stabilize acridinium esters, improve elution of nucleic acids from solid supports. Characterized acridinium esters by HPLC, UV and chemiluminescence.

Research Associate, Department of Microbiology, University of Virginia, 1982 - 1985. Developed new DNA sequencing method similar to Sanger approach, but which leaves functional 3' ends, which can be ligated to produce a set of deletion mutants or can be extended under conditions forcing misincorporation to generate a set of point mutations. Synthesized series of 5'-thymidine triphosphate derivatives containing a 3'-phosphate mono-, di-, or triester group. Showed that these analogs were not substrates for T4 or Klenow DNA polymerase. Developed simple, rapid gel filtration method for purifying and desalting nucleotides. Synthesized an analog of dUTP containing an EDTA group and showed that it can be enzymatically incorporated into DNA.

Assistant Professor, Department of Chemistry, University of Virginia, 1980-1982. Prepared nucleotide derivatives of tubercidin. Characterized allylamine derivatives. Taught biophysical chemistry.

Postdoctoral Research Fellow, Department of Molecular Biophysics and Biochemistry (laboratory of Dr. David C. Ward), Yale University, 1977-1980. Synthesized modified pyrimidines to incorporate in vitro into nucleic acids, using reactions between heavy metals and nucleic acid components. Developed nucleotide analogs used for gene detection in situ. Biotinyl nucleotides now selling commercially.

Predoctoral Fellow, Department of Biophysics (laboratory of Dr. Michael Beer), Johns Hopkins University, Baltimore, Maryland, 1970-1977. Developed multiple heavy atom stains for electron microscopy of nucleic acids.

ACHIEVEMENTS Co-inventor of non-radioactively-labeled nucleotides, including biotinyl nucleotides (U.S. Patents Nos. 4,711,955; 5,328,824; 5,449,767; and 5,476,928). Co-inventor of activated 9-acridinecarboxylic acid chemiluminescent system. Experienced in chemistry of nucleic acids and proteins, especially the synthetic chemistry of nucleotides, peptides, and their oligomers, and in the chemistry of mercury, osmium, and palladium; familiar with NMR, UV-Visible, IR, and fluorescent spectroscopic techniques, and with TLC, HPLC, gel filtration, and ion exchange chromatographic procedures; experienced in the use of DNA polymerases and nucleases. Experienced in detection systems for nucleic acids, especially chemiluminescence. Experienced in chemistry of acridine and acridinium compounds. Experienced with several ELISA enzymes,

including horseradish peroxidase (HRPO), alkaline phosphatase, glucose oxidase, and β -galactosidase.

Publications

- (1) Richardson, F.S., Shillady, D.D., Waldrop, A.A.; A Theoretical Study of Cis-Trans Photoisomerization in the Bis(Glycinato) Platinum(II) Complex, Inorganica Chimica Acta, 5, 279-289 (1971).
- (2) Waldrop, A.A., Beer, M., Marzilli, L.G.; Osmium-labeled Polynucleotides. Incorporation of Additional Heavy Atoms (Mercury) via Ligand Substitution Reactions, Journal of Inorganic Biochemistry, 10, 225-234 (1979).
- (3) Langer P.R., Waldrop, A.A., and Ward, D.C.; Enzymatic Synthesis of Polynucleotides Containing Biotin: Novel Nucleic Acid Affinity Probes, Proc. Natl. Acad. Sci. U.S.A., 78, 6633-6637 (1981).
- (4) Hammond, Philip W.; Wiese, Wendy A.; Waldrop, Alex A., III; Nelson, Norman C.; Arnold, Lyle J., Jr.; Nucleophilic Addition to the 9 Position Of 9-Phenylcarboxylate-10-Methylacridinium Protects Against Hydrolysis of the Ester, J. Biolumin. Chemilumin. 6(1), 35-43, (1991).
- (5) Waldrop, Alex A., III; Fellers, Jonathan; Vary, Calvin P. H.; Chemiluminescent Determination of Hydrogen Peroxide with 9-Acridinecarbonylimidazole and Use in Measurement of Glucose Oxidase and Alkaline Phosphatase Activity, Luminescence 15(3), 168-182, (2000).

Patents and Patent Applications

- (1) Ward, D.C., Langer, P.R., and Waldrop, A.A.; Modified Nucleotides and Methods of Preparing and Using Same, U.S. Patent 4,711,955 (December 8, 1987). (European Pat. Appl. EP 63879 A2)
- (2) Arnold, Lyle J., Waldrop, Alex A., III, Hammond, Philip W.; Protected Chemiluminescent Labels, U. S. Patent # 4,950,613 (Aug. 21, 1990). (European Pat. Appl. EP 330433 A2).
- (3) Ward, D.C., Langer, P.R., and Waldrop, A.A.; Methods of Using Labeled Nucleotides. U.S. Patent #5,328,824 (July 12, 1994).
- (4) Ward, D.C., Langer, P.R., and Waldrop, A.A.; Modified Polynucleotides and Methods of Preparing Same. U.S Patent #5,449,767 (Sept.12, 1995).
- (5) Ward, D.C., Langer, P.R., and Waldrop, A.A.; Modified Nucleotides and Polynucleotides and Complexes Form Therefrom. U.S Patent #5,476,928 (Dec.19, 1995).

- (6) Arnold, Lyle, J., Jr.; Nelson, Norman C.; Reynolds, Mark A.; Waldrop, Alex A., III; Polycationic Supports and Nucleic Acid Purification, Separation and Hybridization. U. S. Patent #5,599,667 (Feb 4, 1997). (European Pat. Appl. EP 281390 A2).
- (7) Waldrop, Alex A., III and Vary, C.P.H., Peroxide-Based Chemiluminescent Assays and Chemiluminescent Compounds Used Therein. Patent pending (Submitted 1997 as Provisional Patent Application).

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Enzymatic synthesis of biotin-labeled polynucleotides: Novel nucleic acid affinity probes

(nucleotide analog/DNA and RNA polymerase/avidin-Sepharose/antibiotin antibody/immunoprecipitation)

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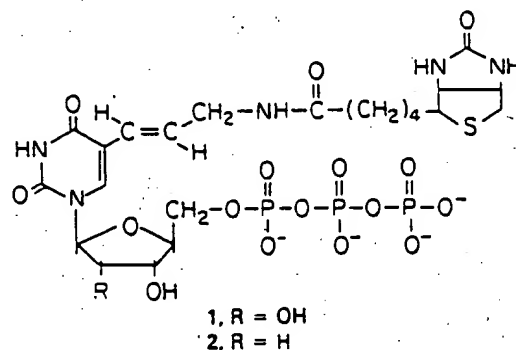
Communicated by Joseph G. Gall, June 29, 1981

ABSTRACT Analogs of dUTP and UTP that contain a biotin molecule covalently bound to the C-5 position of the pyrimidine ring through an allylamine linker arm have been synthesized. These biotin-labeled nucleotides are efficient substrates for a variety of DNA and RNA polymerases *in vitro*. Polynucleotides containing low levels of biotin substitution (50 molecules or fewer per kilobase) have denaturation, reassociation, and hybridization characteristics similar to those of unsubstituted controls. Biotin-labeled polynucleotides, both single and double stranded, are selectively and quantitatively retained on avidin-Sepharose, even after extensive washing with 8 M urea, 6 M guanidine hydrochloride, or 99% formamide. In addition, biotin-labeled polynucleotides can be selectively immunoprecipitated in the presence of anti-biotin antibody and *Staphylococcus aureus* protein A. The unique features of biotin-labeled polynucleotides suggest that they will be useful affinity probes for the detection and isolation of specific DNA and RNA sequences.

Nucleotide analogs that can function as indicator "probes" when incorporated in polynucleotides would be of significant utility in many procedures used in biomedical and recombinant DNA research. When used in conjunction with immunological, histochemical, or affinity detector systems, such reagents could provide suitable alternatives to radioisotopes for the detection, localization, and isolation of nucleic acid components. Biotin (vitamin H) has many features that make it an ideal probe candidate. The interaction between biotin and avidin, a 68,000-dalton glycoprotein from egg white, has one of the highest binding constants ($K_{\text{dis}} = 10^{-15}$) known (1). When avidin is coupled to appropriate indicator molecules (fluorescent dyes, electron-dense proteins, enzymes, or antibodies), minute quantities of biotin can be detected (2-8). The specificity and tenacity of the biotin-avidin complex has been exploited to develop methods for the visual localization of specific proteins, lipids, and carbohydrates on or within cells (for review, see ref. 2). Davidson and associates (9-11) chemically crosslinked biotin to RNA, via cytochrome c or polyamine bridges, and used these RNA-biotin complexes as probes for *in situ* hybridization. The sites of hybridization were visualized in the electron microscope through the binding of avidin-ferritin or avidin-methacrylate spheres. Although this approach to the detection of polynucleotide sequences was successful in the specialized cases examined, a simpler and more general procedure for preparing biotin-substituted nucleic acids was desirable. Biotin directly attached to a nucleotide that functions as an efficient polymerase substrate would be more versatile, both in the experimental protocols and in the detection methods that could be used.

We have synthesized a number of nucleotide analogs that contain potential probe determinants (e.g., biotin, iminobiotin,

and 2,4-dinitrophenyl groups) covalently attached to the pyrimidine or purine ring in the hope that one of them might prove to be a useful affinity reagent. This report describes the synthesis of biotin-labeled derivatives of UTP and dUTP (1 and 2, respectively) that are substrates for RNA or DNA polymerases. The properties of the resulting biotin-substituted polynucleotides appear to satisfy the basic criteria required of a good affinity probe.



MATERIALS AND METHODS

Materials. Standard NTPs were purchased from P-L Biochemicals, and dUTP was obtained from Sigma. Radiolabeled nucleotides were products of New England Nuclear or Amersham Radiochemicals. *Escherichia coli* DNA polymerase I, both holoenzyme and Klenow fragment, was obtained from Boehringer Mannheim; restriction enzymes were from New England BioLabs or Bethesda Research Laboratories. The following enzymes and reagents were gifts: T7 RNA polymerase and T7 DNA (J. Coleman); herpes simplex DNA polymerase (B. Francke); L1210 and HeLa cell DNA polymerases α and β (H. S. Alladeen); avian myeloblastosis reverse transcriptase (RNA-dependent DNA nucleotidyltransferase) (S. Weissman); murine and calf thymus RNA polymerase II (R. Roeder); *E. coli* RNA polymerase (P. Farnam); and rabbit anti-biotin serum (F. Harmon).

Synthesis of 1 (Bio-UTP) and 2 (Bio-dUTP). *Mercuration step.* The 5-mercured derivatives of UTP and dUTP were prepared by a modification of the procedure of Dale *et al.* (12). UTP (570 mg, 1.0 mmol) or dUTP (554 mg, 1.0 mmol) in 100 ml of 0.1 M sodium acetate, pH 6.0, was treated with mercuric acetate (1.59 g, 5.0 mmol). The solution was heated at 50°C for 4 hr and then cooled on ice. Lithium chloride (392 mg, 9.0 mmol)

Abbreviations: AA-UTP and AA-dUTP, 5-(3-aminolallyl) uridine and deoxyuridine triphosphates, respectively; Bio-UTP and Bio-dUTP, 5-allylaminobiotin-labeled UTP and dUTP, respectively; Bio-RNA and Bio-DNA, biotin-labeled RNA and DNA, respectively; MVM, minute virus of mouse; RF, replicative form.

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was added, and the solution was extracted six times with equal volumes of ethyl acetate to remove excess HgCl_2 . The efficiency of the extraction process was monitored by estimating the mercuric ion concentration in the organic layer by using 4,4'-bis(dimethylamino)-thiobenzophenone (13). The extent of nucleotide mercuriation, determined spectrophotometrically by following the iodination of an aliquot of the aqueous solution (14), was routinely 90–100%. The nucleotide products in the aqueous layer, which often became cloudy during the ethyl acetate extraction, were precipitated by the addition of 3 vol of ice-cold ethanol and collected by centrifugation. The precipitate was washed twice with cold absolute ethanol and once with ethyl ether and then air dried. These products were used for the synthesis of the 5-(3-amino)allyl nucleotides without further purification.

Synthesis of 5-(3-amino)allyluridine and deoxyuridine 5' triphosphates (AA-UTP and AA-dUTP). Organomercurial compounds can be alkylated or arylated under mild conditions by reaction with olefins in the presence of a palladium catalyst (15). Bergstrom and associates (16, 17) have recently used this procedure for the synthesis of C-5-substituted pyrimidine nucleosides. We have also adopted this general synthetic approach for introducing the allylamine linker arm. The mercurated nucleotides were dissolved in 0.1 M sodium acetate, pH 5.0, and adjusted to 20 mM (A_{267} , 200 units/ml). A fresh 2.0 M solution of allylamine (Aldrich) was prepared by slowly adding 1.5 ml of allylamine (13.3 M) to 8.5 ml of ice-cold 4 M acetic acid. Three milliliters (6.0 mmol) of the neutralized allylamine stock was added to 25 ml (0.5 mmol) of nucleotide solution. One nucleotide equivalent of K_2PdCl_4 (163 mg, 0.5 mmol; Alfa-Ventron, Danvers, MA) in 4 ml of water was then added to initiate the reaction; the solution gradually turned black and metal (Hg and Pd) deposits appeared on the walls of the reaction vessel. After standing at room temperature for 18–24 hr, the reaction mixture was passed through a 0.45- μm membrane filter (Nalgene) to remove most of the remaining metal precipitate. The yellow filtrate was diluted 1:5 with H_2O and applied to a 100-ml column of DEAE-Sephadex A-25 (Pharmacia). After washing with 1 column vol of 0.1 M sodium acetate, pH 5.0, the products were eluted by using a 1-liter linear gradient (0.1–0.6 M) of sodium acetate, pH 8–9, or Et_3NHCO_3 , pH 7.5. The desired product was in the major UV-absorbing peak, which eluted between 0.30 and 0.35 M salt. Because spectral analysis showed that this peak contained several products, final purification was achieved by reverse-phase high-pressure liquid chromatography on columns of Partisil-ODS2, using either 0.5 M $(\text{NH}_4)_3\text{PO}_4$, pH 3.3 (analytical separations), or 0.5 M Et_3NHOAc , pH 4.3 (preparative separations), as eluents. AA-UTP and AA-dUTP were the last peaks to elute from the column and they were cleanly resolved from three as-yet unidentified contaminants. The characterization of the (3-amino)allyl nucleotides by proton NMR, elemental, spectral, and chromatographic analyses will be presented in detail elsewhere. These studies clearly showed that the (3-amino)allyl substituent is attached to the C-5 position of the pyrimidine ring and that the olefinic protons are in the *trans* configuration.

Conversion of AA-UTP or AA-dUTP to Bio-UTP and Bio-dUTP. Biotinyl-N-hydroxysuccinimide ester was prepared from biotin (Sigma) as described (3). AA-UTP- $4\text{H}_2\text{O}$ (70 mg, 0.1 mmol) or AA-dUTP- H_2O (63 mg, 0.1 mmol) in 20 ml of 0.1 M sodium borate, pH 8.5, was treated with the ester (34.1 mg, 0.1 mmol) in 2 ml of dimethylformamide. The reaction mixture was left at room temperature for 4 hr and then loaded directly onto a 30-ml column of DEAE-Sephadex A-25 previously equilibrated with 0.1 M Et_3NHCO_3 , pH 7.5. The column was eluted with a 400-ml linear gradient (0.1–0.9 M) of Et_3NHCO_3 . Frac-

tions containing bio-dUTP or bio-UTP, which eluted at 0.55–0.65 M Et_3NHCO_3 , were desalted by rotary evaporation in the presence of methanol and then dissolved in water. Occasionally, a slightly cloudy solution was obtained; this turbidity, due to a contaminant in some Et_3NHCO_3 solutions, was removed by filtration through a 0.45- μm filter. For long-term storage, the nucleotides were converted to sodium salts by briefly stirring the solution in the presence of Dowex 50 (Na^+). After filtration, the nucleotide was precipitated by the addition of 3 vol of cold ethanol, washed with ethyl ether, dried at reduced pressure over sodium hydroxide pellets, and stored in a desiccator at -20°C . For immediate use, the nucleotide solution was made 20 mM in Tris-HCl, pH 7.5, and adjusted to a final nucleotide concentration of 5 mM. Stock solutions were stored at -20°C .

Analysis. Bio-dUTP: Calcd. for $\text{C}_{22}\text{H}_{30}\text{N}_5\text{O}_{19}\text{P}_3\text{SNa}_4\cdot\text{H}_2\text{O}$: C, 29.80; H, 3.38; N, 7.89; P, 10.47; S, 3.61. Found: C, 30.14; H, 3.22; N, 7.63; P, 10.31; S, 3.70. Bio-UTP: Calcd. for $\text{C}_{22}\text{H}_{30}\text{N}_5\text{O}_{19}\text{P}_3\text{SNa}_4\cdot 3\text{H}_2\text{O}$: C, 29.15; H, 3.19; N, 7.45; P, 9.89; S, 3.41. Found: C, 28.76; H, 3.35; N, 7.68; P, 9.81; S, 3.32. The spectral properties of bio-dUTP and bio-UTP at pH 7.5 [λ_{max} 289 nm ($\epsilon = 7100$); λ_{max} 240 nm ($\epsilon = 10,700$); λ_{min} 262 nm ($\epsilon = 4300$)] reflect the presence of an exocyclic double bond conjugated with the pyrimidine ring. These nucleotides also give a strong positive reaction (an orange-red color) when treated with *p*-dimethylaminocinnamaldehyde in ethanolic sulfuric acid, a procedure used for biotin quantitation (18). However, in contrast to AA-dUTP and AA-UTP, they do not give a positive ninhydrin reaction.

RESULTS

Bio-UTP and Bio-dUTP have been synthesized. These analogs were then tested for their ability to function as substrates for a series of purified nucleic acid polymerases *in vitro*. As shown in Fig. 1, Bio-dUTP is an excellent substrate for *E. coli* DNA polymerase I using either the nick-translation protocol of Rigby *et al.* (19) or the "gap-filling" reaction described by Bourguignon *et al.* (21). Although it is incorporated at an initial rate that is only 30–40% of that of the control reaction with TTP, the final specific activities (and the extent of polymerization) that can be achieved are essentially the same. Bio-dUTP is also a substrate for bacteriophage T4 DNA polymerase, DNA polymerases α and β from murine (A-9) and human (HeLa) cells, and the DNA polymerase of herpes simplex virus, with incorporation efficiencies similar to that of *E. coli* DNA polymerase I (not shown). In addition, Bio-dUTP will support DNA synthesis in a nuclear replication system prepared from baby hamster kidney cells infected with herpes simplex virus (unpublished data). In contrast, Bio-dUTP does not function as a substrate for avian myeloblastosis virus reverse transcriptase under standard incubation conditions using mRNA-oligo(dT), minute virus of mouse (MVM) DNA, or poly(dA)-oligo(dT) as template-primer complexes.

The ribonucleotide analog, Bio-UTP, can substitute for UTP in reactions catalyzed by the RNA polymerases of *E. coli* and bacteriophage T7 (Fig. 2), although with a lower efficiency than that of any DNA polymerase/Bio-dUTP system. Furthermore, Bio-UTP is utilized poorly, if at all, by the eukaryotic RNA polymerases we have examined (HeLa cell RNA polymerase III, calf thymus RNA polymerase II, and mouse L-cell RNA polymerase II). Although the limited range of substrate function precludes the use of Bio-UTP in the direct enzymatic biotin labeling of eukaryotic transcripts *in vivo*, biotin-labeled RNA (Bio-RNA) probes can be prepared *in vitro* by using appropriate DNA templates and *E. coli* RNA polymerase or by 3'-end labeling methods using RNA ligase and biotin-labeled pUp (not shown).

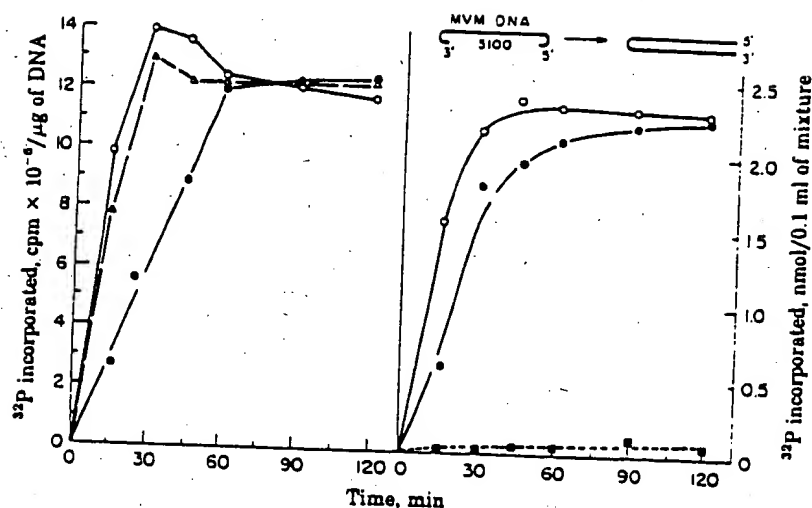


FIG. 1. Bio-dUTP is a substrate for *E. coli* DNA polymerase I. (A) A Phage DNA was nick translated *in vitro* with DNA polymerase I holoenzyme as described (19). Reactions used [^{32}P]dATP (1.35 μM , 400 Ci/mmol) and either 20 μM TTP (○), 20 μM Bio-dUTP (●), or 10 μM TTP/10 μM Bio-dUTP (△). (B) Minute virus of mouse (MVM) DNA, a 5-kilobase single-stranded DNA with terminal hairpin duplexes (20), was converted to a double-stranded form by reaction with DNA polymerase I (Klenow fragment) as described (21). The three-nucleotide reaction (■; no TTP) contained dCTP, dGTP, and [^{32}P]dATP (50 $\mu\text{Ci}/\mu\text{mol}$) at 0.1 mM. TTP (○) and Bio-dUTP (●) reactions were supplemented with the appropriate triphosphate at a final concentration of 0.1 mM.

The enzymatic polymerization of nucleotides containing biotin was not monitored directly because neither Bio-dUTP or Bio-UTP were radiolabeled. However, two lines of evidence show that the biotin-labeled nucleotides are incorporated. The first is that polynucleotides synthesized in the presence of biotin-labeled nucleotides are selectively retained when chromatographed over avidin-Sepharose affinity columns. For example, normal DNA, nick translated with TTP, dCTP, dGTP, and [^{32}P]dAMP, is quantitatively eluted from avidin-Sepharose by the addition of 0.5 M NaCl. In contrast, the majority of nick-translated biotin-labeled DNA (Bio-DNA) remains bound to the resin even after extensive washing with high salt, urea, guanidine-HCl, formamide, 2 mM biotin, or 50 mM NaOH (Table 1). The small fraction of radiolabel eluted by these washing conditions is not retained when it is applied to the resin a second time, suggesting that this radioactivity is associated with DNA fragments that are free of biotin substitution. Because the pBR322 DNA used in this experiment had $\approx 5\%$ of its thymidine residues substituted by Bio-dUMP (based on picomoles of [^{32}P]dAMP incorporated in the nick-translation reaction), it is clear that only a few molecules of biotin per kilobase of DNA are necessary for irreversible binding to avidin-Sepharose. Indeed, when the "sticky" ends of Simian virus 40 DNA (linearized by treatment with *Eco*RI) are filled in by using Bio-dUTP and *E. coli* DNA polymerase Klenow fragment, the DNA is retained on avidin-Sepharose (unpublished data). Thus, four biotin molecules or fewer per five kilobases of DNA are sufficient for selective retention.

The second line of evidence for biotin substitution is that only polynucleotides synthesized in the presence of biotin-labeled nucleotides are immunoprecipitated when treated with purified anti-biotin antibodies and then with formalin-fixed *Staphylococcus* (Table 2). Although the amount of biotin-labeled polymer found in the immune precipitate is dependent on the antibody concentration and time of incubation, under optimum conditions, >90% of the product can be immunoprecipitated, even when present in subnanogram quantities. Significantly, the results in Tables 1 and 2 show that the biotin molecule can be recognized by avidin and anti-biotin antibodies when the DNA is still in a double-stranded form. Parallel experiments (not shown) indicate that biotin-labeled DNA-RNA hybrids and RNA duplexes behave similarly. These observations suggest that immunological and affinity methods could be used for probe detection (or isolation) following standard hybridization procedures.

To determine whether biotin-substituted polynucleotides

were suitable for use as hybridization probes, the denaturation and renaturation characteristics of several biotin-labeled DNA and RNA polymers were examined. As shown in Table 3, the melting temperature of DNA duplexes decreases as the Bio-dUMP content of the polymer increases. A parallel analysis of RNA duplexes and DNA-RNA hybrids (not shown) indicates that they respond similarly. However, a pronounced decrease in melting temperature occurs only in heavily substituted polymers (e.g., poly(dA-dBio-U)) and even then the degree of coop-

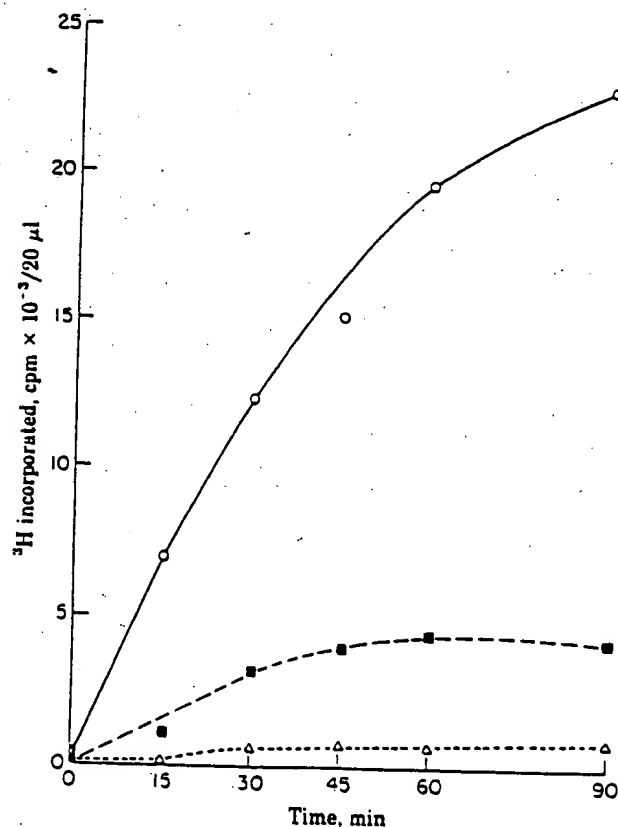


FIG. 2. Bio-UTP is a substrate for T7 RNA polymerase. Reaction mixtures (0.15 ml) were 40 mM Tris-HCl, pH 8.0/30 mM MgCl_2 /10 mM 2-mercaptoethanol/0.4 mM ATP/0.4 mM GTP/0.4 mM [^3H]CTP (100 $\mu\text{Ci}/\mu\text{mol}$)/0.4 mM UTP or Bio-UTP containing 7.5 μg of T7 DNA and 3.2 μg of T7 RNA polymerase. Aliquots (20 μl) were removed at the indicated times and acid precipitated onto glass-fiber filters. Curves: ○, UTP; ■, Bio-UTP; △, three-nucleotide reaction; no UTP or Bio-UTP.

Table 1. Selective retention of biotin-labeled DNA on avidin-Sepharose

	% DNA retained on resin	
	Bio-DNA (5%)*	Control
Load	100	100
Eluent		
0.5 M NaCl	100	0.1
1.0 M NaCl	99.7	<0.01
8 M Urea	100	<0.01
6 M Guanidine-HCl	95.2	<0.01
99% Formamide	94.7	<0.01
2 mM Biotin	97.6	<0.01
50 mM NaOH	89.5	<0.01

Avidin-Sepharose was prepared by coupling avidin to cyanogen bromide-activated Sepharose 4B essentially as described (22). Columns containing 0.2 ml of resin were equilibrated with 10 mM Tris-HCl/0.20 M NaCl, pH 7.5, and the DNA samples (3×10^6 cpm per load; 2×10^7 cpm/ μ g) were applied in 0.2 ml of the same buffer. The columns were washed with 1.0 ml of loading buffer and then treated with 2.0 ml of eluent. The % DNA retained on the resin was calculated by using the formula (cpm of DNA loaded - cpm eluted) \div cpm loaded, as determined by Cerenkov counting.

* pBR322 DNA labeled with [32 P]dAMP by nick translation in which 5% of the TMP residues have been replaced by Bio-dUMP.

erativity and the extent of hyperchromicity observed during denaturation are virtually identical to that of control polymers. Thus, pBR322 or λ DNAs that have been nick translated to introduce ≈ 20 biotin molecules per kilobase have melting temperatures similar to those of their biotin-free counterparts. Even MVM replicative form (RF) DNA in which every TMP residue in one strand (≈ 1250 in 5 kilobases) is replaced by Bio-dUMP has a melting temperature that is only 5°C less than that of the unsubstituted DNA. Of greater significance is the observation that lightly labeled DNA probes hybridize in solution at essentially the same rate as biotin-free probes (Fig. 3). Furthermore, 32 P-labeled biotin-substituted pBR322 DNA has the same degree of specificity and autoradiographic signal intensity as control biotin-free pBR322 DNA when used as a hybridization probe for detecting bacterial colonies that contain the plasmid (data not shown). These results indicate that a substantial number of biotin-labeled nucleotides can be introduced into a

Table 2. Selective immunoprecipitation of Bio-DNA with anti-biotin IgG and Staphylococcus

DNA	Antibody	Radioactivity, cpm	
		Precipitate	Supernatant
Control	—	70	4867
Control	Anti-Bio IgG	87	5197
Control	Nonimmune IgG	55	5107
Bio-DNA	—	53	3886
Bio-DNA	Anti-Bio IgG	3347	736
Bio-DNA	Nonimmune IgG	60	3900

Immunoprecipitation of DNA samples was done essentially as described (23). Biotin-labeled and control pBR322 DNAs labeled with [32 P]dAMP by nick translation (specific activity, 2×10^7 cpm/ μ g) were treated with 100 μ l of formalin-fixed Staphylococcus (IgG Sorb, The Enzyme Center) in water for 10 min at room temperature. The supernatants from these reaction mixtures were incubated at 4°C for 1 hr without serum, with nonimmune rabbit serum, or with rabbit anti-biotin affinity purified from serum provided by Fred Harmon. Immune complexes were precipitated by the addition of 50 μ l of IgG Sorb. After 10 min at room temperature, the mixtures were centrifuged, and the pellets were washed three times with 30 mM Tris-HCl/150 mM NaCl/0.05% Nonidet P-40, pH 7.5, and analyzed by Cerenkov counting.

Table 3. Effect of biotin substitution on the thermal denaturation of DNA duplexes

DNA	Bio-dUMP content (% total nucleotides)	T_m , °C
Control pBR322	—	80
Biotin-labeled pBR322	2.0	79
Control MVM RF	—	69
Biotin-labeled MVM RF	12.5	64
poly(dA-dT)	—	62
poly(dA-dBio-U)	50.0	47

pBR322 DNAs were prepared by nick translation and thermally denatured in 10 mM Tris-HCl/50 mM NaCl/1.0 mM EDTA, pH 7.5. MVM RF DNAs were prepared as described in the legend to Fig. 1, and melting profiles were determined in 10 mM Tris-HCl/1.0 mM EDTA, pH 7.5. poly(dA-dT) and poly(dA-dBio-U) were prepared from *E. coli* DNA polymerase I reactions primed by poly(dA-dT) as described (24), and melting profiles were determined in 10 mM Tris-HCl/0.10 M NaCl/1.0 mM EDTA, pH 7.5.

nucleic acid probe without significantly altering its hybridization characteristics.

Several additional properties of biotin-labeled polynucleotides are worth noting at this point. First, phenol extraction should be avoided whenever possible during purification of Bio-DNA or Bio-RNA because heavily substituted polymers are extracted into the phenol layer and even lightly or moderately substituted ones (e.g., nick-translated DNAs) can often be retained at the phenol/H₂O interface. Second, because the mass of Bio-dUMP is about twice that of TMP, extensive substitution can appreciably increase the overall mass of the polymer. For example, biotin-labeled MVM RF DNA (Fig. 1B) and restriction fragments derived from it migrate more slowly in agarose gels than their biotin-free counterparts (Fig. 4). Finally, incorporation of a biotin-labeled nucleotide into a restriction endonuclease recognition site may prevent enzymatic cleavage.

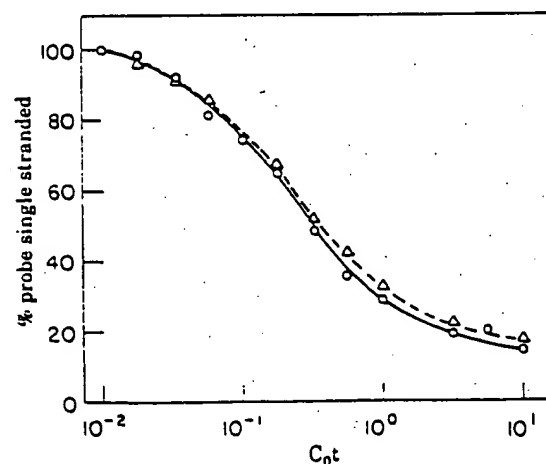


FIG. 3. Effect of biotin substitution on the reassociation rate of *E. coli* DNA. Sheared *E. coli* DNA was nick translated by using [α - 32 P]dATP and either TTP or Bio-dUTP to a specific activity of 1.3×10^6 cpm/ μ g; the Bio-DNA probe had $\approx 5.5\%$ of its TMP residues replaced by Bio-dUMP. The probes were heat denatured and hybridized at 37°C to a 220-fold excess of denatured nonradiolabeled *E. coli* DNA in 50% formamide/0.30 M NaCl/0.03 M sodium citrate, pH 7.0. Aliquots (10 μ l) were removed at various times and diluted into 100 μ l of 0.05 M sodium acetate, pH 5.0/0.05 M NaCl/1.0 mM ZnCl₂. Mung bean nuclease (2 units; P-L Biochemicals) was added, and the mixture was incubated at 42°C for 15 min. The amount of 32 P-labeled probe made resistant to the single-strand-specific nuclease was determined by acid precipitation onto glass-fiber filters. C_0t , initial concentration of DNA (moles of nucleotide/liter) \times time (sec). \circ , Control DNA; Δ , Bio-DNA probe.

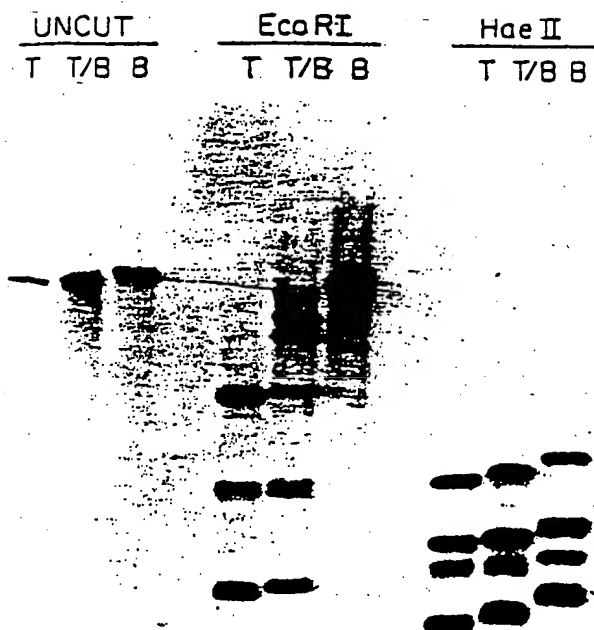


FIG. 4. Effect of biotin substitution on restriction endonuclease cleavage of DNA. ³²P-Labeled MVM RF DNAs prepared as described in the legend to Fig. 1B were cleaved with *Eco*RI and *Hae* II as described (20), and the samples were subjected to electrophoresis in a 1.4% agarose gel. Lanes: T, DNA gap filled with TTP as a substrate; T/B, DNA gap filled in the presence of equimolar concentrations of TTP and Bio-dUTP; B, DNA gap filled with Bio-dUTP. The faint bands in the *Hae* II lanes reflect a trace contamination of the enzyme with *Hae* III.

When Bio-dUMP is in the recognition site of *Eco*RI (G-A-A-T-T-C), the DNA is totally refractory to this enzyme although it remains sensitive to digestion by *Hae* II (PuG-C-C-CPy) (Fig. 4).

DISCUSSION

Our data demonstrate that Bio-dUTP and Bio-UTP are used as substrates by a number of nucleic acid polymerases, albeit at somewhat lower rates than the parent compounds, TTP and UTP. This provides a simple and rapid procedure for synthesizing chemically stable biotin-substituted polymers that hybridize specifically and efficiently to complementary sequences either in solution or bound to solid supports. Because polynucleotides containing a limited number of biotin molecules (50 or fewer per kb) hybridize with kinetics similar to those of unlabeled controls, standard hybridization protocols need be modified little if at all. The observation that Bio-DNA or Bio-RNA, and nonbiotinized sequences that hybridize to them, are selectively retained on avidin-Sepharose columns or immunoprecipitated by the addition of anti-biotin antibodies and *Staphylococcus* is significant in several regards. First, these results suggest that biotin-labeled polymers can be used in conjunction with appropriate immunofluorescent, immunohistochemical, or affinity reagents for detecting or localizing specific sequences in

chromosomes, cells, tissue sections, and blots. Our studies have led to the development of a rapid method of gene mapping by *in situ* hybridization that uses rabbit anti-biotin antibody and fluorescein-labeled goat anti-rabbit IgG to identify the loci of hybridized Bio-DNA probes and a histochemical procedure for detecting biotin-labeled sequences on nitrocellulose filters that uses antibody-alkaline phosphatase conjugates (unpublished data). Second, the ability to synthesize immunogenic DNAs (and to a lesser extent RNAs) enzymatically, both in purified *in vitro* systems and in crude cell lysates, may allow the use of immunoprecipitation techniques. Finally, because the interaction between biotin-labeled polynucleotide probes and avidin-Sepharose is essentially irreversible, it should be possible to develop refined protocols for enriching (or deleting) specific gene sequences from complex mixtures in a fashion analogous to that reported by Manning *et al.* (25). Although further studies are obviously required, our results indicate that enzymatically biotin-labeled polynucleotides can function as nucleic acid affinity reagents.

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which are covered with two different, but adjacent, layers of a chromatographic support.

coupled neutral pump A coupled pump in which the movement of one ion across the membrane must be linked to the movement of another ion, of equal valence, in the opposite direction.

coupled pump A pump for the transport of one solute across a membrane that also drives the transport of a second solute across the same membrane in the opposite direction and in such a fashion that the transport of the second solute is physically dependent on the pump.

coupled reactions An endergonic and an exergonic reaction that are linked energetically; the endergonic reaction is driven by the exergonic reaction which occurs simultaneously and which shares a common intermediate with the endergonic reaction, such that the overall free energy change for the coupled reactions is negative. The ultimate coupling requirement is that the free energy change for each step in the mechanism (usually $\Delta G'$, at pH 7.0) must be ≤ 0 . *Aka* energetically coupled reactions; energy coupling.

coupled transcription-translation The process, characteristic of prokaryotes, in which transcription and translation proceed simultaneously; the mRNA is being translated into protein before transcription of DNA into the mRNA has been completed.

coupled transport A transport system in which the movement of one solute across the membrane must be linked to the movement of a second solute across the same membrane but in the opposite direction.

coupling 1. The linking of aerobic respiration, specifically the operation of the electron transport system, to the synthesis of ATP. 2. The tendency of linked genes to be inherited together on the same chromosome. 3. CHANNELING.

coupling constant The separation between any two bands of multiple peaks in nuclear magnetic resonance; it is proportional to the magnitude of the spin-spin coupling. *Sym* J.

coupling factors A group of proteins that are required for the coupling of ATP synthesis to the operation of the electron transport system either in mitochondrial oxidative phosphorylation or in chloroplast photosynthesis. The mitochondrial coupling factor 1 is now called F_1 -ATPase. *See also* F_0F_1 -ATPase.

coupling inhibition UNCOMPETITIVE INHIBITION.

covalent bond A bond formed between two atoms and consisting of one or more shared pairs of electrons such that one electron in a

pair is donated by each of the two bonded atoms. *See also* coordinate covalent bond.

covalent catalysis Catalysis that requires the formation of a covalent enzyme-substrate intermediate.

covalent chromatography A column chromatographic technique in which a chemical reagent is linked covalently to the solid support. When a sample is passed through the column, the reagent reacts with, and binds covalently, the substance of interest. An additional chemical reaction then releases the substance from the support and permits its elution from the column, thereby restoring the initial form of the support.

covalent circle *See* circular covalent; covalently closed circle.

covalent enzyme-substrate complex ENZYME-SUBSTRATE COMPOUND.

covalent extension The initiation of DNA replication in which the leading strand is covalently attached to a parental strand as in the rolling circle replication.

covalent intermediate 1. A substance formed during covalent catalysis such as the intermediate formed in the transaminase reaction. 2. A covalently linked, high-energy intermediate that, according to the chemical coupling hypothesis, functions in the coupling aspect of oxidative phosphorylation.

covalent labeling AFFINITY LABELING.

covalently circular *See* circular covalent.

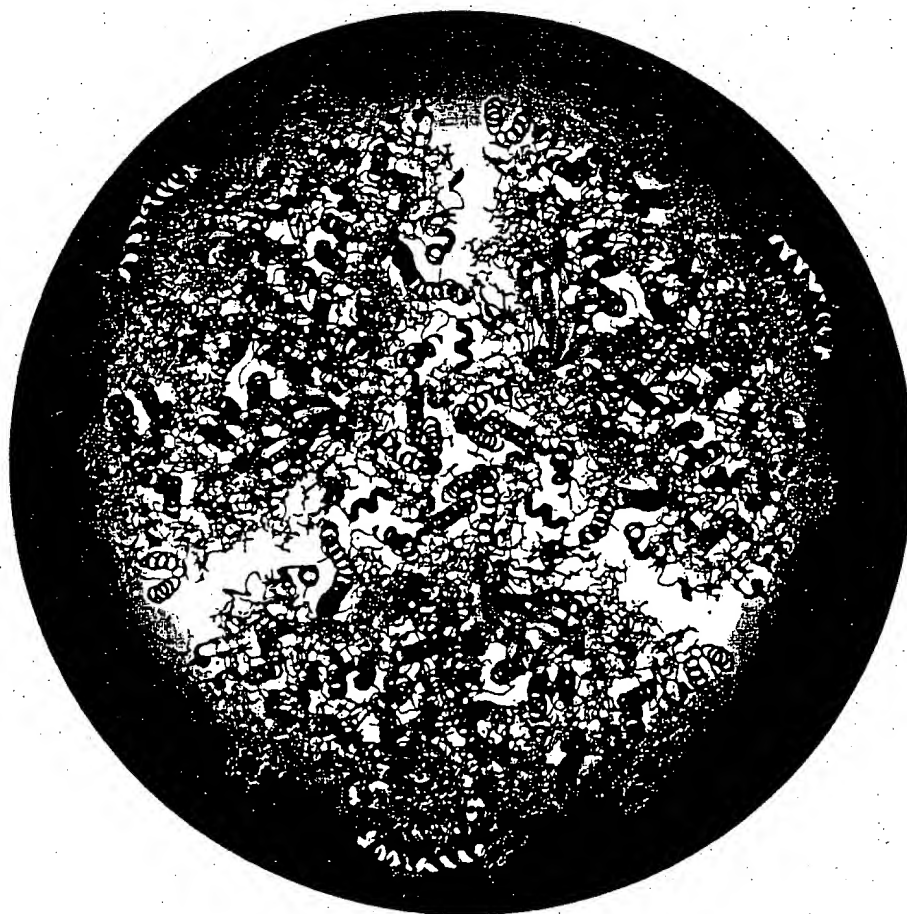
covalently closed circle A circular, double-stranded, DNA molecule in which each single strand is an unbroken, uninterrupted circle.

covalently modified enzyme A regulatory enzyme that has the capacity of having its catalytic activity modified through chemical alteration of the molecule which, in turn, is catalyzed by other enzymes. The enzyme-catalyzed phosphorylation and dephosphorylation of the enzyme phosphorylase is an example.

covalent orbital An orbital that functions in the bonding of a low-spin complex.

covalent structure analysis The determination of the covalent bonds that describe the arrangement of monomers in a macromolecule; the bonds that describe the amino acid sequence and the location of disulfide bonds in a protein, or those that describe the nucleotide sequence in a nucleic acid are examples.

covariance The average product of the deviations from the respective means for all pairs of values for the variables X and Y ; the average of $(X - \bar{X})(Y - \bar{Y})$ for all pairs of values of X and Y , where \bar{X} and \bar{Y} are the means for the X values and Y values, respectively.



Biochemistry

Third Edition

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About the Cover

"Sun Catcher." The structure of the trimeric Photosystem I from the thermophilic cyanobacterium *Synechococcus elongatus*. This protein complex captures light energy from the sun and converts it into the chemical energy of an oxidation-reduction reaction. Image provided by Norbert Krauss, Petra Fromme, Wolfram Saenger, Horst Tobias Witt, and Patrick Jordan, of the Institute for Crystallography, Free University of Berlin and the Max Volmer Institute for Biophysical Chemistry and Biochemistry at the Technical University Berlin.

can turn, fold, and coil in the three dimensions of space to establish a specific, highly ordered architecture that is an identifying characteristic of the given protein molecule (Figure 1.11).

Weak Forces Maintain Biological Structure and Determine Biomolecular Interactions

Covalent bonds hold atoms together so that molecules are formed. In contrast, **weak chemical forces** or **noncovalent bonds** (hydrogen bonds, van der Waals forces, ionic interactions, and hydrophobic interactions) are intramolecular or intermolecular attractions between atoms. None of these forces, which typically range from 4 to 30 kJ/mol, are strong enough to bind free atoms together (Table 1.3). The average kinetic energy of molecules at 25°C is 2.5 kJ/mol, so the energy of weak forces is only several times greater than the dissociating tendency due to thermal motion of molecules. Thus, these weak forces create interactions that are constantly forming and breaking at physiological temperature, unless by cumulative number they impart stability to the structures generated by their collective action. These weak forces merit further discussion because their attributes profoundly influence the nature of the biological structures they build.

Van der Waals Attractive Forces Play an Important Role in Biomolecular Interactions

Van der Waals forces are the result of induced electrical interactions between closely approaching atoms or molecules as their negatively charged electron clouds fluctuate instantaneously in time. These fluctuations allow attractions to occur between the positively charged nuclei and the electrons of nearby atoms. Van der Waals interactions include dipole-dipole interactions, whose interaction energies decrease as $1/r^3$; dipole-induced dipole interactions, which fall off as $1/r^3$; and induced dipole-induced dipole interactions, often called **dispersion** or **London dispersion forces**, which diminish as $1/r^6$. Dispersion forces contribute to the attractive intermolecular forces between all molecules, even those without permanent dipoles, and are thus generally more important than dipole-dipole attractions. Van der Waals attractions operate only over a very limited interatomic distance (0.3 to 0.6 nm) and are an effective bonding interaction at physiological temperatures only when a number of atoms in a molecule can interact with several atoms in a neighboring molecule. For this to occur, the atoms on interacting molecules must pack together neatly. That is,

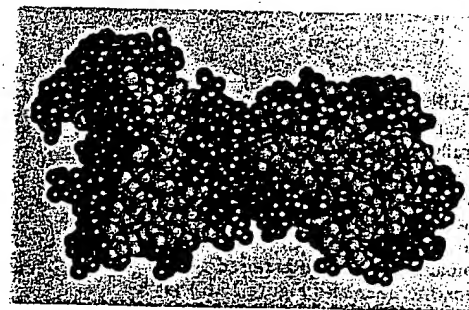


FIGURE 1.11 Three-dimensional space-filling representation of part of a protein molecule, the antigen-binding domain of immunoglobulin G (IgG). IgG is a major type of circulating antibody. Each of the spheres represents an atom in the structure.

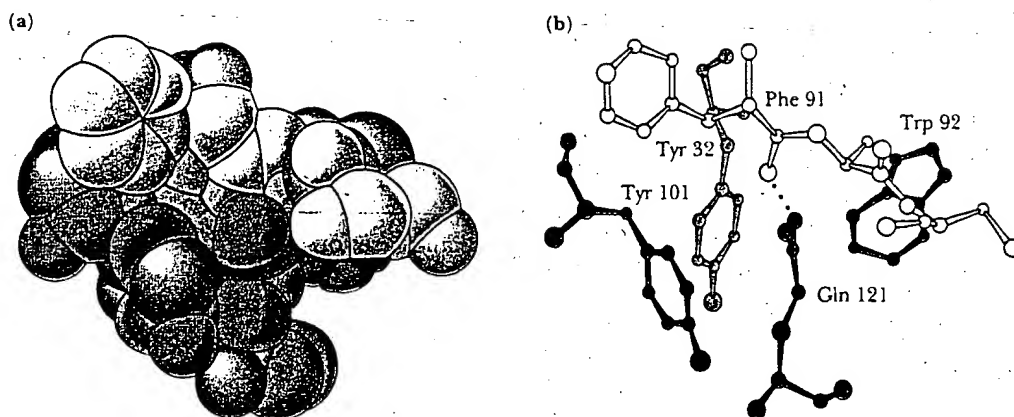
A *dipole* is any structure with equal and opposite electrical charges separated by a small distance.

Table 1.3

Weak Chemical Forces and Their Relative Strengths and Distances

Force	Strength (kJ/mol)	Distance (nm)	Description
Van der Waals interactions	0.4–4.0	0.3–0.6	Strength depends on the relative size of the atoms or molecules and the distance between them. The size factor determines the area of contact between two molecules: The greater the area, the stronger the interaction.
Hydrogen bonds	12–30	0.3	Relative strength is proportional to the polarity of the H bond donor and H bond acceptor. More polar atoms form stronger H bonds.
Ionic interactions	20	0.25	Strength also depends on the relative polarity of the interacting charged species. Some ionic interactions are also H bonds: $\text{—NH}_3^+ \dots \text{—OOC—}$
Hydrophobic interactions	<40	—	Force is a complex phenomenon determined by the degree to which the structure of water is disordered as discrete hydrophobic molecules or molecular regions coalesce.

FIGURE 1.12 Van der Waals packing is enhanced in molecules that are structurally complementary. Gln¹²¹ represents a surface protuberance on the protein lysozyme. This protuberance fits nicely within a pocket (formed by Tyr¹⁰¹, Tyr³², Phe⁹¹, and Trp⁹²) in the antigen-binding domain of an antibody raised against lysozyme. (See also Figure 1.16.) (a) A space-filling representation. (b) A ball-and-stick model. (From Amit, A. G., et al., 1986. Three-dimensional structure of an antigen-antibody complex at 2.8 Å resolution. *Science* 233:747–753, figure 5.)



their molecular surfaces must possess a degree of structural complementarity (Figure 1.12).

At best, van der Waals interactions are weak and individually contribute 0.4 to 4.0 kJ/mol of stabilization energy. However, the sum of many such interactions within a macromolecule or between macromolecules can be substantial. For example, model studies of heats of sublimation show that each methylene group in a crystalline hydrocarbon accounts for 8 kJ, and each C—H group in a benzene crystal contributes 7 kJ of van der Waals energy per mole. Calculations indicate that the attractive van der Waals energy between the enzyme lysozyme and a sugar substrate that it binds is about 60 kJ/mol.

When two atoms approach each other so closely that their electron clouds interpenetrate, strong repulsion occurs. Such *repulsive* van der Waals forces follow an inverse 12th-power dependence on r ($1/r^{12}$), as shown in Figure 1.13. Between the repulsive and attractive domains lies a low point in the potential curve. This low point defines the distance known as the **van der Waals contact distance**, which is the interatomic distance that results if only van der Waals forces hold two atoms together. The limit of approach of two atoms is determined by the sum of their van der Waals radii (Table 1.4).

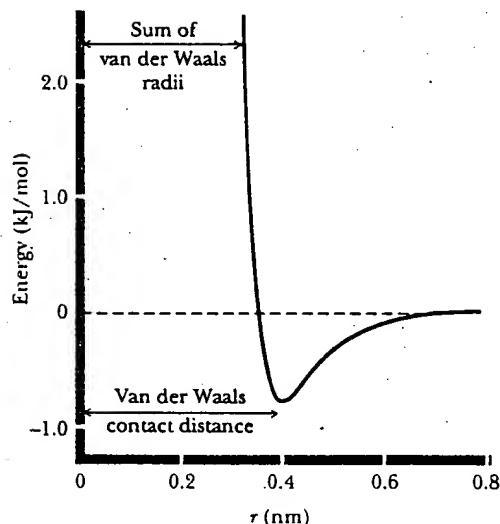


FIGURE 1.13 The van der Waals interaction energy profile as a function of the distance, r , between the centers of two atoms. The energy was calculated using the empirical equation $U = B/r^{12} - A/r^6$. (Values for the parameters $B = 11.5 \times 10^{-66}$ kJnm¹²/mol and $A = 5.96 \times 10^{-3}$ kJnm⁶/mol for the interaction between two carbon atoms are from Levitt, M., 1974. Energy refinement of hen egg-white lysozyme. *Journal of Molecular Biology* 82:393–420.)







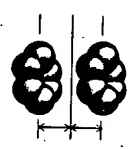
Hydrogen Bonds Are Important in Biomolecular Interactions

Hydrogen bonds form between a hydrogen atom covalently bonded to an electronegative atom (such as oxygen or nitrogen) and a second electronegative atom that serves as the hydrogen bond acceptor. Several important biological examples are given in Figure 1.14. Hydrogen bonds, at a strength of 12 to 30 kJ/mol, are stronger than van der Waals forces and have an additional property: H bonds are cylindrically symmetrical and tend to be highly directional, forming straight bonds between donor, hydrogen, and acceptor atoms. Hydrogen bonds are also more specific than van der Waals interactions because they require the presence of complementary hydrogen donor and acceptor groups.

Ionic Interactions Ionic interactions are the result of attractive forces between oppositely charged polar functions, such as negative carboxyl groups and positive amino groups (Figure 1.15). These electrostatic forces average about 20 kJ/mol in aqueous solutions. Typically, the electrical charge is radially distributed, so these interactions may lack the directionality of hydrogen bonds or the precise fit of van der Waals interactions. Nevertheless, because the opposite charges are restricted to sterically defined positions, ionic interactions can impart a high degree of structural specificity.

Table 1.4

Radii of the Common Atoms of Biomolecules

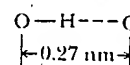
Atom	Van der Waals Radius (nm)	Covalent Radius (nm)	Atom Represented to Scale
H	0.1	0.037	
C	0.17	0.077	
N	0.15	0.070	
O	0.14	0.066	
P	0.19	0.096	
S	0.185	0.104	
Half-thickness of an aromatic ring	0.17	—	

The strength of electrostatic interactions is highly dependent on the nature of the interacting species and the distance, r , between them. Electrostatic interactions may involve **ions** (species possessing discrete charges), **permanent dipoles** (having a permanent separation of positive and negative charge), and **induced dipoles** (having a temporary separation of positive and negative charge induced by the environment). Between two ions, the strength of interaction diminishes as $1/r$. The interaction energy between permanent dipoles falls off as $1/r^3$, whereas the energy between an ion and an induced dipole falls off as $1/r^4$.

Hydrophobic Interactions Hydrophobic interactions result from the strong tendency of water to exclude nonpolar groups or molecules (see Chapter 2). Hydrophobic interactions arise not so much because of any intrinsic affinity of nonpolar substances for one another (although van der Waals forces do promote the weak bonding of nonpolar substances), but because water molecules prefer the stronger interactions that they share with one another, compared to their interaction with nonpolar molecules. Hydrogen-bonding interactions between polar water molecules can be more varied and numerous if nonpolar molecules come together to form a distinct organic phase. This phase separation raises the entropy of water because fewer water molecules are arranged in orderly arrays around individual nonpolar molecules. It is these preferential interactions between water molecules that “exclude” hydrophobic substances from aqueous solution and drive the tendency of nonpolar molecules to cluster together. Thus, nonpolar regions of biological macromolecules are often buried in the molecule’s interior to exclude them from the aqueous milieu. The formation of oil droplets as hydrophobic nonpolar lipid molecules coalesce in the presence of water is an approximation of this phenomenon. These tendencies have important consequences in the creation and maintenance of the macromolecular structures and supramolecular assemblies of living cells.

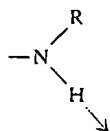
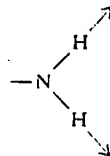
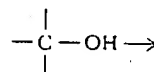
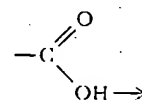
(a) H bonds Bonded atoms	Approximate bond length*
O—H---O	0.27 nm
O—H---O ⁻	0.26 nm
O—H---N	0.29 nm
N—H---O	0.30 nm
⁺ N—H---O	0.29 nm
N—H---N	0.31 nm

*Lengths given are distances from the atom covalently linked to the H to the atom H bonded to the hydrogen:

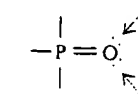
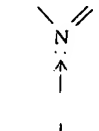
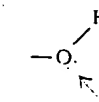
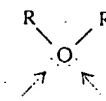
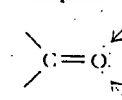


(b) Functional groups that are important H-bond donors and acceptors:

Donors

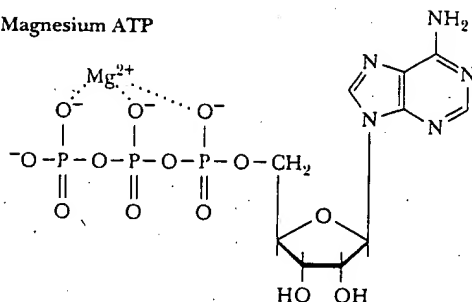


Acceptors

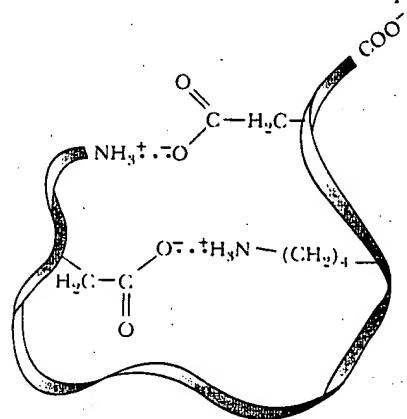


Biochemistry Now™ ANIMATED FIGURE 1.14
Some of the biologically important H bonds and functional groups that serve as H bond donors and acceptors. See this figure animated at <http://chemistry.brookscole.com/ggb3>


Magnesium ATP



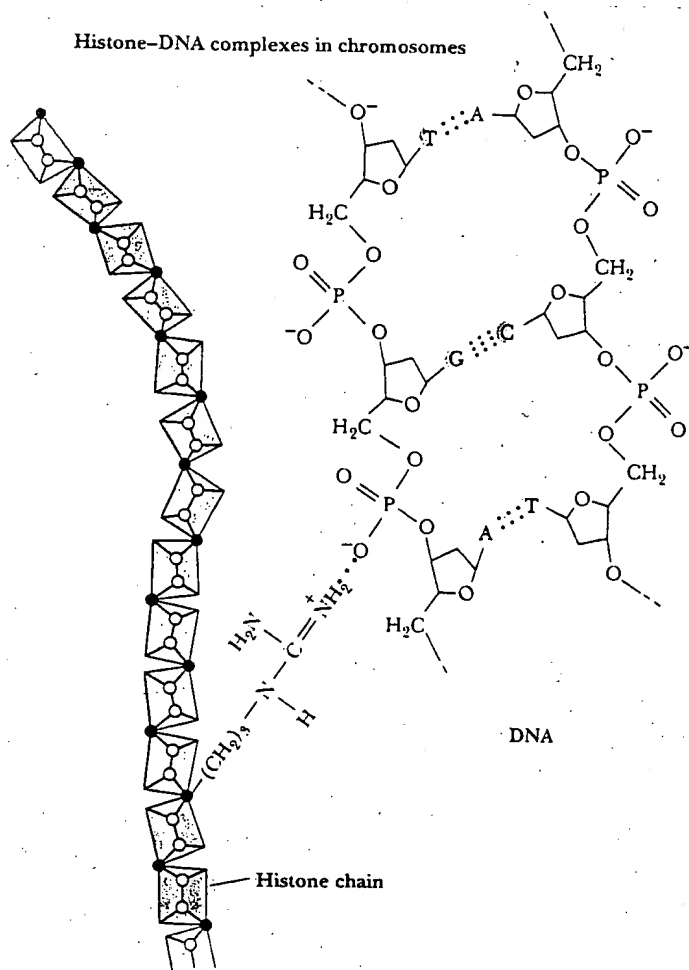
Intramolecular ionic bonds between oppositely charged groups on amino acid residues in a protein



Protein strand

Biochemistry  Now™ ANIMATED FIGURE 1.15
Ionic bonds in biological molecules. See this figure
animated at <http://chemistry.brookscole.com/ggb3>

Histone-DNA complexes in chromosomes

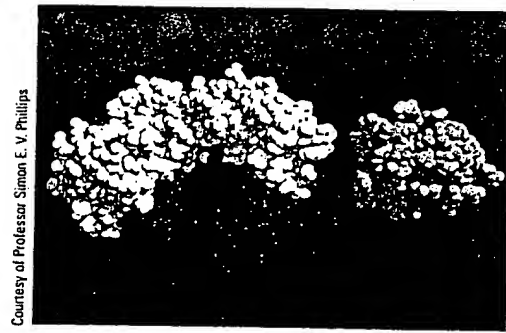


The Defining Concept of Biochemistry Is "Molecular Recognition Through Structural Complementarity"

Structural complementarity is the means of recognition in biomolecular interactions. The complicated and highly organized patterns of life depend on the ability of biomolecules to recognize and interact with one another in very specific ways. Such interactions are fundamental to metabolism, growth, replication, and other vital processes. The interaction of one molecule with another, a protein with a metabolite, for example, can be most precise if the structure of one is complementary to the structure of the other, as in two connecting pieces of a puzzle or, in the more popular analogy for macromolecules and their **ligands**, a lock and its key (Figure 1.16). *This principle of structural complementarity is the very essence of biomolecular recognition.* Structural complementarity is the significant clue to understanding the functional properties of biological systems. Biological systems from the macromolecular level to the cellular level operate via specific molecular recognition mechanisms based on structural complementarity: A protein recognizes its specific metabolite, a strand of DNA recognizes its complementary strand, sperm recognize an egg. All these interactions involve structural complementarity between molecules.

Biomolecular Recognition Is Mediated by Weak Chemical Forces

Weak chemical forces underlie the interactions that are the basis of biomolecular recognition. It is important to realize that because these interactions are sufficiently weak, they are readily reversible. Consequently, biomolecular inter-

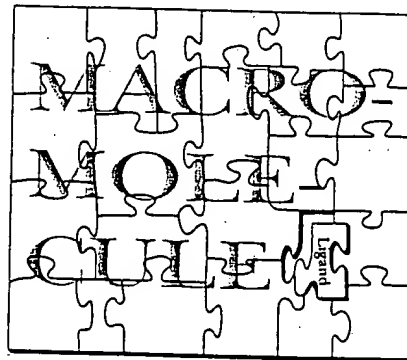


(a)



(b)

Puzzle



Lock and key

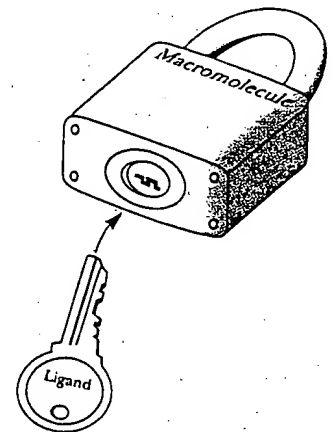


FIGURE 1.16 Structural complementarity: the pieces of a puzzle, the lock and its key, a biological macromolecule and its ligand—an antigen–antibody complex. (a) The antigen on the right (green) is a small protein, lysozyme, from hen egg white. The part of the antibody molecule (IgG) shown on the left in blue and yellow includes the antigen-binding domain. (b) This domain has a pocket that is structurally complementary to a surface protuberance (Gln¹³¹, shown in red between antigen and antigen-binding domain) on the antigen. (See also Figure 1.12.)

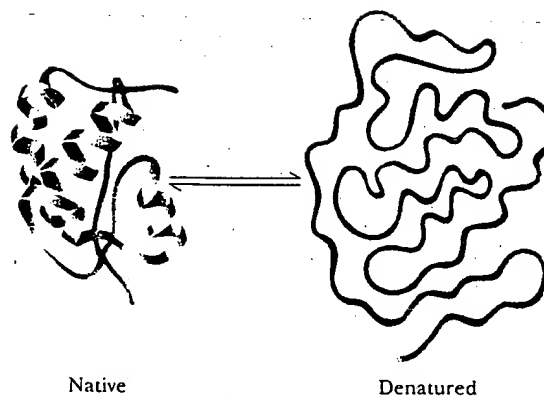
actions tend to be transient; rigid, static lattices of biomolecules that might paralyze cellular activities are not formed. Instead, a dynamic interplay occurs between metabolites and macromolecules, hormones and receptors, and all the other participants instrumental to life processes. This interplay is initiated upon specific recognition between complementary molecules and ultimately culminates in unique physiological activities. Biological function is achieved through mechanisms based on structural complementarity and weak chemical interactions.

This principle of structural complementarity extends to higher interactions essential to the establishment of the living condition. For example, the formation of supramolecular complexes occurs because of recognition and interaction between their various macromolecular components, as governed by the weak forces formed between them. If a sufficient number of weak bonds can be formed, as in macromolecules complementary in structure to one another, larger structures assemble spontaneously. The tendency for nonpolar molecules and parts of molecules to come together through hydrophobic interactions also promotes the formation of supramolecular assemblies. Very complex subcellular structures are actually spontaneously formed in an assembly process that is driven by weak forces accumulated through structural complementarity.

BiochemistryNow™ Go to BiochemistryNow and click BiochemistryInteractive to explore the structure of immunoglobulin G, centering on the role of weak intermolecular forces in controlling structure.

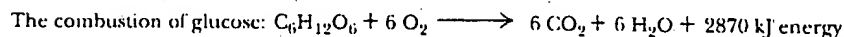
Weak Forces Restrict Organisms to a Narrow Range of Environmental Conditions

Because biomolecular interactions are governed by weak forces, living systems are restricted to a narrow range of physical conditions. Biological macromolecules are functionally active only within a narrow range of environmental conditions, such as temperature, ionic strength, and relative acidity. Extremes of these conditions disrupt the weak forces essential to maintaining the intricate structure of macromolecules. The loss of structural order in these complex macromolecules, so-called **denaturation**, is accompanied by loss of function (Figure 1.17). As a consequence, cells cannot tolerate reactions in which large amounts of energy are

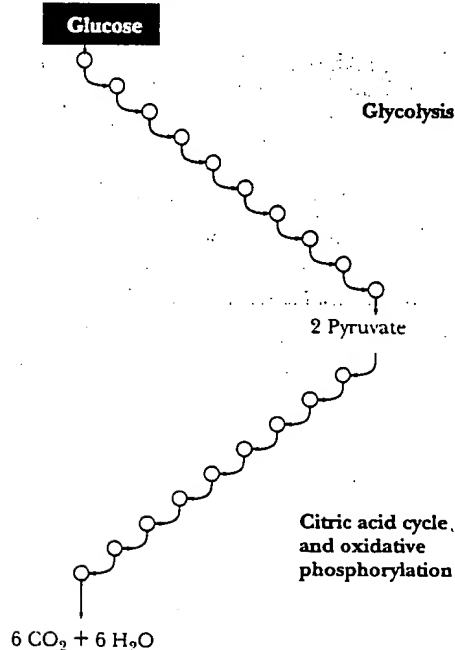


BiochemistryNow™ ANIMATED FIGURE 1.17
Denaturation and renaturation of the intricate structure of a protein. See this figure animated at <http://chemistry.brookscole.com/ggb3>.

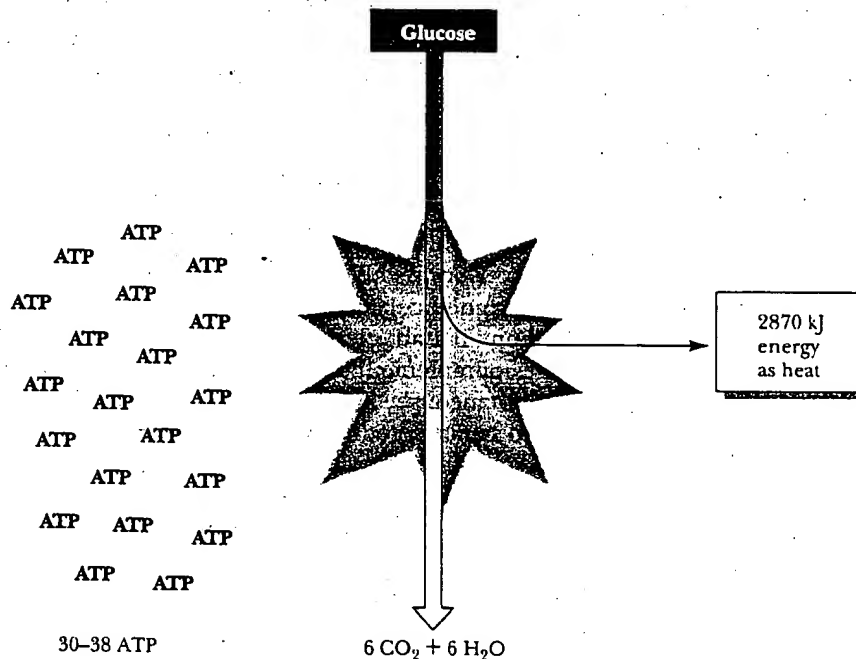
released, nor can they generate a large energy burst to drive energy-requiring processes. Instead, such transformations take place via sequential series of chemical reactions whose overall effect achieves dramatic energy changes, even though any given reaction in the series proceeds with only modest input or release of energy (Figure 1.18). These sequences of reactions are organized to provide for the release of useful energy to the cell from the breakdown of food or to take such energy and use it to drive the synthesis of biomolecules essential to the living state. Collectively, these reaction sequences constitute cellular **metabolism**—the ordered reaction pathways by which cellular chemistry proceeds and biological energy transformations are accomplished.



(a) In an aerobic cell



(b) In a bomb calorimeter



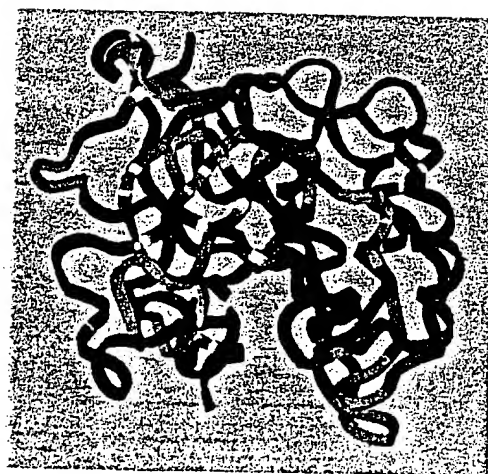
Biochemistry Now™ **ACTIVE FIGURE 1.18** Metabolism is the organized release or capture of small amounts of energy in processes whose overall change in energy is large. (a) For example, the combustion of glucose by cells is a major pathway of energy production, with the energy captured appearing as 30 to 38 equivalents of ATP, the principal energy-rich chemical of cells. The ten reactions of glycolysis, the nine reactions of the citric acid cycle, and the successive linked reactions of oxidative phosphorylation release the energy of glucose in a stepwise fashion and the small “packets” of energy appear in ATP. (b) Combustion of glucose in a bomb calorimeter results in an uncontrolled, explosive release of energy in its least useful form, heat. **Test yourself on the concepts in this figure at <http://chemistry.brookscole.com/ggb3>**

Enzymes Catalyze Metabolic Reactions

The sensitivity of cellular constituents to environmental extremes places another constraint on the reactions of metabolism. The rate at which cellular reactions proceed is a very important factor in maintenance of the living state. However, the common ways chemists accelerate reactions are not available to cells; the temperature cannot be raised, acid or base cannot be added, the pressure cannot be elevated, and concentrations cannot be dramatically increased. Instead, biomolecular catalysts mediate cellular reactions. These catalysts, called **enzymes**, accelerate the reaction rates many orders of magnitude and, by selecting the substances undergoing reaction, determine the specific reaction that takes place. Virtually every metabolic reaction is catalyzed by an enzyme (Figure 1.19).

Metabolic Regulation Is Achieved by Controlling the Activity of Enzymes Thousands of reactions mediated by an equal number of enzymes are occurring at any given instant within the cell. Metabolism has many branch points, cycles, and interconnections, as a glance at a metabolic pathway map reveals (Figure 1.20). All these reactions, many of which are at apparent cross-purposes in the cell, must be fine-tuned and integrated so that metabolism and life proceed harmoniously. The need for metabolic regulation is obvious. This metabolic regulation is achieved through controls on enzyme activity so that the rates of cellular reactions are appropriate to cellular requirements.

Despite the organized pattern of metabolism and the thousands of enzymes required, cellular reactions nevertheless conform to the same thermodynamic principles that govern any chemical reaction. Enzymes have no influence over energy changes (the thermodynamic component) in their reactions. Enzymes only influence reaction rates. Thus, cells are systems that take in food, release waste, and carry out complex degradative and biosynthetic reactions essential to their survival while operating under conditions of essentially constant temperature and pressure and maintaining a constant internal environment (**homeostasis**) with no outwardly apparent changes. *Cells are open thermodynamic systems exchanging matter and energy with their environment and functioning as highly regulated isothermal chemical engines.*



Biochemistry Now™ ANIMATED FIGURE 1.19

Carbonic anhydrase, a representative enzyme, and the reaction that it catalyzes. Dissolved carbon dioxide is slowly hydrated by water to form bicarbonate ion and H^+ :



At 20°C, the rate constant for this uncatalyzed reaction, k_{uncat} is 0.03/sec. In the presence of the enzyme carbonic anhydrase, the rate constant for this reaction, k_{cat} is 10^6 /sec. Thus, carbonic anhydrase accelerates the rate of this reaction 3.3×10^7 times. Carbonic anhydrase is a 29-kD protein. See this figure animated at <http://chemistry.brookscole.com/ggb3>

1.5 What Is the Organization and Structure of Cells?

All living cells fall into one of two broad categories—**prokaryotic** and **eukaryotic**. The distinction is based on whether the cell has a nucleus. Prokaryotes are single-celled organisms that lack nuclei and other organelles; the word is derived from *pro* meaning “prior to” and *karyot* meaning “nucleus.” In conventional biological classification schemes, prokaryotes are grouped together as members of the kingdom Monera, represented by bacteria and cyanobacteria (formerly called blue-green algae). The other four living kingdoms are all eukaryotes—the single-celled Protists, such as amoebae, and all multicellular life forms, including the Fungi, Plant, and Animal kingdoms. Eukaryotic cells have true nuclei and other organelles such as mitochondria, with the prefix *eu* meaning “true.”

The Evolution of Early Cells Gave Rise to Eubacteria, Archaea, and Eukaryotes

For a long time, most biologists believed that eukaryotes evolved from the simpler prokaryotes in some linear progression from simple to complex over the course of geological time. However, contemporary evidence favors the view that

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